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Mechanisms of Replication-Deficient Vaccinia Virus/T7 RNA Polymerase Hybrid Expression: Effect of T7 RNA Polymerase Levels and α-Amanitin

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Components of the eukaryotic vaccinia virus/T7 RNA polymerase hybrid expression system were assessed using recombinant and nonrecombinant forms of modified vaccinia Ankara (MVA), a replication-deficient vaccinia virus strain. Recombinant MVA virus expressing T7 RNA polymerase (Wyatt, L. S., Moss, B., and Rozenblatt, S. (1995). *Virology* 210, 202–205) stimulated high levels of expression from a T7 promoter–chloramphenicol acetyltransferase (CAT) reporter. Most, but not all, of the virally induced expression was T7 RNA polymerase and T7 promoter dependent, with no viral enhancement of translation of T7 transcripts. The efficacy of supplying T7 RNA polymerase expression from nonviral sources was evaluated using a self-amplifying T7 RNA polymerase autogene or an inducible T7 RNA polymerase expression vector. The latter modes yielded CAT activity dependent on T7 RNA polymerase expression; however, expression required viral factors independent of T7 RNA polymerase and did not reach that attained using the recombinant virus. In further experiments, MVA-induced T7 RNA polymerase expression was upregulated by α-amanitin, an inhibitor of eukaryotic polymerases. This indicates that MVA/T7 RNA polymerase hybrid expression may be rendered still more efficient by ameliorating transcriptional interference due to an α-amanitin-sensitive eukaryotic factor(s).

INTRODUCTION

Bacteriophage enzymes such as T7 RNA polymerase (T7RP) have been used with considerable success as components of eukaryotic expression systems (Moss *et al.,* 1990; Deng *et al.,* 1991; Gao and Huang, 1993; Chen *et al.,* 1994; Ward *et al.,* 1995). In particular, T7RP-mediated expression is highly efficient in eukaryotic cells when coupled with vaccinia virus infection (Elroy-Stein and Moss, 1990). Vaccinia virus/T7RP hybrid systems allow levels of expression up to 30% of cellular RNA (Fuerst and Moss, 1989) or approximately 10% of cellular protein (Elroy-Stein and Moss, 1990). An attractive feature of the system is that transcription with the single subunit phage polymerase is less dependent than host polymerases on endogenous eukaryotic factors and therefore is less susceptible to promoter effects inherent in eukaryotic transcription complexes (Holter *et al.,* 1991). Furthermore, vaccinia virus-associated cytoplasmic expression using T7RP may circumvent the obligatory translocation of transfected DNA to the nucleus required of conventional eukaryotic promoters, thus bypassing a major barrier to eukaryotic expression (Zabner *et al.,* 1995). Such aspects of T7 expression may allow expression less contingent on cell type, cellular metabolism, or growth state than conventional mammalian expression vectors.

Typically, when recombinant vaccinia viruses are used to deliver target genes and/or T7RP, amplification of target gene copy number, and hence enhancement of expression, occurs as a consequence of both viral genome replication and spreading viral infection (Moss *et al.,* 1990). However, such vaccinia virus vectors encode their own polymerase and transcription factors, have pleiotropic effects on eukaryotic transcription and translation, and cause rapid cytopathic effects (Moss, 1990). Thus, it would be very desirable to find conditions which maintain the high level of expression typical of recombinant viruses, but in which infection levels and viral effects are reduced or eliminated. In this regard, a recent advance has been made through the use of an attenuated vaccinia virus (modified vaccinia virus Ankara, MVA), which does not give rise to infectious particles in human cells and causes fewer cytopathic effects in cell monolayers (Sutter and Moss, 1992). Expression using this replication-deficient virus in a recombinant form designed to deliver T7RP is comparable to, or higher than, that of replicating virus (Sutter *et al.,* 1995; Wyatt *et al.,* 1995), indicating that viral propagation is not absolutely necessary to generate high expression. However, viral DNA amplification of target genes as contained in the attenuated MVA virus remains a factor.

Here, we report two experimental approaches which dissociate the coding of T7RP from recombinant virus, providing instead the requisite T7RP activity from a non-

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viral source. First, a self-amplifying T7RP autogene (the T7RP gene under control of its own promoter) (Dubendorf and Studier, 1991; Chen *et al.,* 1994; Gao *et al.,* 1994) was constructed which yields high expression in vaccinia virus-infected cells without the use of a recombinant virus. Second, HeLa cell lines were constructed which express inducible endogenous T7RP activity; these cells likewise yield high levels of expression following infection with nonrecombinant virus. These approaches have allowed us to address an unresolved issue in the use of such vaccinia-based systems, namely, the extent to which viral infection per se, as opposed to the level of virus-encoded T7RP, enhances the expression of transfected genes. Further, by dissociating T7RP activity from recombinant virus, it has been possible to analyze in greater detail how each of the components of the vaccinia virus/T7RP system (virus, T7RP, and target gene) contributes to overall expression.

RESULTS

T7 promoter/T7RP-dependent expression using a dual CMV/T7 promoter

The MVA strain of vaccinia virus was chosen for these studies, since this strain aborts its replicative cycle at a late-stage packaging step in human cells (Sutter and Moss, 1992). Expression is thus achieved without propagation of infectious virus. To test the ability of the replication-deficient MVA virus to enhance expression, HeLa cells were infected with either unmodified MVA or a recombinant MVA strain modified to express T7RP (Wyatt *et al.,* 1995) (generous gifts from L. Wyatt and B. Moss, National Institutes of Health). The cells were then transiently transfected with identical plasmids containing the CAT gene under control of the CMV promoter with or without an included T7 promoter (Fig. 1). Infection by MVA/T7RP resulted in a multifold increase in chloramphenicol acetyltransferase (CAT) activity upon transfection of pCMV[P_{T7}]/CAT (Fig. 1, lane 1 vs lanes 3–7) which was not observed for pCMV[$-P_{T7}$]/CAT (Fig. 1, lane 10). Lysates from MVA/T7RP-infected cells transfected with pCMV[P_{T7}]/CAT could be diluted up to 200-fold (Fig. 1, lanes 4–7) before producing comparable acetylated chloramphenicol levels achieved by pCMV[$-P_{T7}$]/CAT (Fig. 1, lane 10). The dual CMV and T7 promoters produced 440- and 3.5-fold (440 divided by 126) higher levels of CAT expression than the CMV[$-P_{T7}$] or T7 expression units alone, respectively, in MVA/T7RP-infected cells (Table 1). This represents 1200-fold higher activity than when no CAT DNA was transfected (Fig. 1, lane 1 vs lanes 3–7). Interestingly, an MVA-associated increase in CMV expression was also evident which was T7RP and T7 promoter-independent (Fig. 1, lane 8 vs lanes 9 and 10).

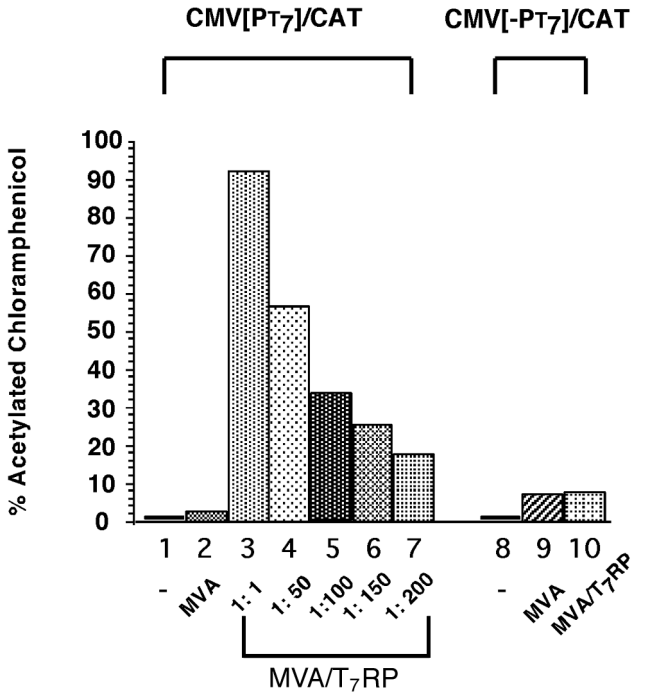

FIG. 1. Effect of MVA and MVA/T7RP infection on pCMV/CAT expression. HeLa cells were transfected with 1 μg pCMV/CAT DNA with (lanes 1–7) or without (lanes 8–10) a T7 promoter in the absence of infection (lanes 1 and 8) or in the presence of either 2 multiplicities of infection (m.o.i.) MVA (lanes 2 and 9) or MVA/T7RP (lanes 3–7, 10). CAT assays were performed for 2 h with 5 μg (lanes 1–3, 8–10) or the indicated dilutions of 5 μg (lanes 3–7) of cell lysate. The products were separated by thin layer chromatography (TLC), visualized by autoradiography, and quantified by phosphorimaging.

T7RP-dependent expression using T7 expression vectors

An attractive feature of the vaccinia virus/T7RP expression system is its capacity for cytoplasmic expression (Moss *et al.,* 1990). Plasmids containing the CAT gene under control of a T7 promoter, but devoid of eukaryotic promoters, were constructed which contained 5′ and 3′ sequences designed to enhance expression of T7 transcripts in eukaryotic cells. The 5′ UTR from the encephalomyocarditis (EMC) virus precedes the CAT gene and confers cap-independent translation by acting as an internal ribosome entry site (IRES) (Jang *et al.,* 1989). The presence of a 5′ EMC IRES has been shown to increase eukaryotic expression of T7 transcripts 4- to 10-fold in vaccina virus-infected cells (Elroy-Stein *et al.,* 1989). Similarly, the CAT gene is followed by a portion of the 3′ UTR from the *Xenopus laevis* β-globin gene and a polynucleotide tract of 30 adenine residues. This region has been shown to increase eukaryotic expression of T7 RNA transcripts, presumably by increasing their stability (Malone *et al.,* 1989).

HeLa cells were transiently transfected with the P_{T7} expression vector, with or without the CAT gene, to test the functionality of the T7 expression unit (Fig. 2). Although no detectable CAT activity was observed for the

TABLE 1

Quantitation of CAT Activity Using Various Constructs in Infected Cells

Plasmid	Infection Agent (Relative activity)[a]	
	MVA	MVA/T7RP
pCMV[$-P_{T7}$]/CAT	1	1
pCMV[$+P_{T7}$]/CAT	0.4	440
pCMV[$-P_{T7}$]/CAT	1	1
pCITE/CAT/$X\beta gA_{30}$[b]	0.5	126
pCITE/CAT/$X\beta gA_{30}$ + AutoT7RP[c]	8	35
pCITE/CAT/$X\beta gA_{30}$ + AutoT7RP + pCMV[$-P_{T7}$]/T7RP	8	35

[a] HeLa cells were transiently transfected with the indicated plasmids and CAT assays were performed as described. The relative activity is expressed as the fold induction compared with that produced with pCMV[$-P_{T7}$]/CAT alone. The values in the top two lines are derived from data depicted graphically in Fig. 1, while the bottom four lines include additional data from the same experiment.

[b] The pCITE/CAT/$X\beta gA_{30}$ expression unit consists of P_{T7}/EMC/CAT/$X\beta gA_{30}$.

[c] The AutoT7RP expression unit consists of P_{T7}/EMC/T7RP/$X\beta gA_{30}$.

empty vector under any condition of infection (Fig. 2, lanes 1–3) or for P_{T7}/EMC/CAT/$X\beta gA_{30}$ in the absence of infection (Fig. 2, lane 4), MVA infection alone resulted in increased CAT activity for P_{T7}/EMC/CAT/$X\beta gA_{30}$ DNA (Fig. 2, lane 5), which was greatly enhanced after MVA/T7RP infection (Fig. 2, lane 6). Quantitation of CAT expression for P_{T7}/EMC/CAT/$X\beta gA_{30}$ DNA after MVA/T7RP infection showed 126-fold higher expression than pCMV[$-P_{T7}$]/CAT (Table 1).

Effect of viral infection on T7 CAT RNA expression

Inasmuch as vaccinia virus does not influence the uptake or trafficking of transfected DNA (Zabner *et al.,* 1995), the increased expression from P_{T7}/EMC/CAT/$X\beta gA_{30}$ DNA after MVA/T7RP infection should be due either to amplification of transfected DNA or increased DNA transcription and/or translation of T7 transcripts. Vaccinia virus-mediated amplification of DNA appears unlikely since vaccinia-mediated increases in T7 expression occur in the presence of cytosine-β-D-arabinofuranoside (AraC), an inhibitor of DNA synthesis (Elroy-Stein and Moss, 1990). To eliminate translation as a possibility, *in vitro* synthesized EMC/CAT/$X\beta gA_{30}$ RNA was transfected into HeLa cells, and CAT expression was assayed under adjusted conditions of infection (Fig. 2). Compared to a tRNA control, which showed no detectable CAT activity under any condition of infection (Fig. 2, lanes 7–9), transfected EMC/CAT/$X\beta gA_{30}$ RNA showed comparable levels of CAT activity in uninfected cells (Fig. 2, lane 10) or after MVA (Fig. 2, lane 11) or MVA/T7RP infection (Fig. 2, lane 12). In each of these cases, expression did not approach that of P_{T7}/EMC/T7RP/$X\beta gA_{30}$ DNA (Fig. 2, lane 6).

T7RP autogene

Increasing the multiplicity of infection of MVA/T7RP has been shown to increase expression of P_{T7} target genes ((Wyatt *et al.,* 1995) and data not shown). However, it is unknown whether such increases are due to increases in viral factors or the polymerase enzyme. To dissociate MVA-associated versus T7RP-dependent effects on expression, a number of P_{T7}/EMC/T7RP/$X\beta gA_{30}$ autogenes were constructed (see Materials and Methods) so as to produce high levels of polymerase in the absence of MVA/T7RP infection. Candidate autogenes and pCMV[$-P_{T7}$]/T7RP were tested for activity by expression of CAT from a cotransfected P_{T7}/CAT reporter gene after MVA infection. For active autogenes, T7RP activity as measured by CAT expression increased linearly as the amount of transfected P_{T7}/EMC/T7RP/$X\beta gA_{30}$ autogene DNA increased from 0 to 10 μg under identical MVA infection levels (Fig. 3, lanes 1–6). However, increasing autogene levels further became inhibitory (data not shown). Addition of the T7RP autogene to MVA/T7RP-infected cells inhibited CAT expression by approximately fourfold (126 divided by 35, Table 1). Although an active pCMV[$-P_{T7}$]/T7RP construct was also made, its cotransfection together with the autogene did not give increased expression over that observed with the autogene alone (Table 1).

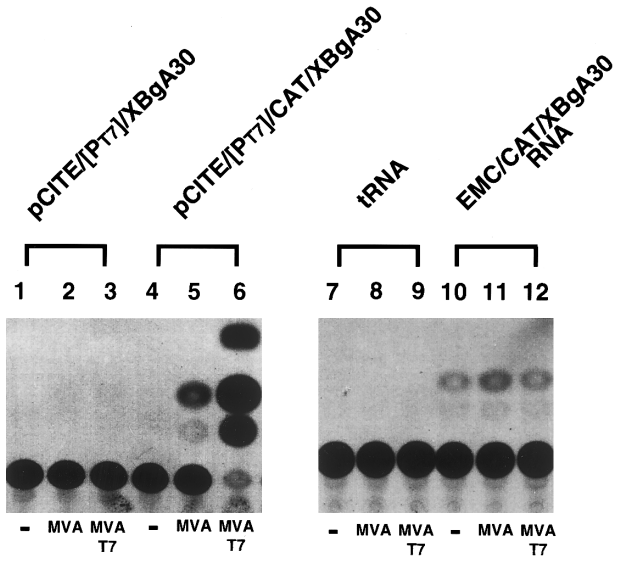

FIG. 2. MVA and MVA/T7RP infection increases CAT expression from DNA but not RNA transfections. HeLa cells were transfected with 1 μg of the T7 promoter/EMC IRES-bearing pCITE-2a plasmid without (lane 1–3) or with (lanes 4–6) CAT after no (lanes 1 and 4), 2 m.o.i. MVA (lanes 2 and 5), or 2 m.o.i. MVA/T7RP (lanes 3 and 6) infection. HeLa cells were transfected with tRNA (lanes 7–9) or EMC/CAT/$X\beta gA_{30}$ RNA (lanes 10–12) in the absence of infection (lanes 7 and 10) or the presence of 2 m.o.i. MVA (lanes 8 and 11) or 2 m.o.i. MVA/T7RP (lanes 9 and 12) infection. Cell extracts were analyzed for CAT activity as described using 5 μg of lysate for 30 min, and the resultant autoradiograph is shown.

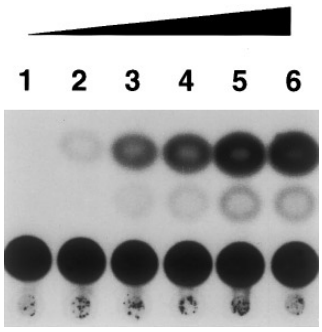

FIG. 3. Increasing addition of T7RP autogene results in increasing CAT expression. HeLa cells were infected with 2 m.o.i. MVA and transiently cotransfected with 1 μg of T7 promoter/EMC/CAT/*X*βgA$_{30}$-bearing plasmids with 0 (lane 1), 0.01 (lane 2), 0.05 (lane 3), 0.1 (lane 4), 0.5 (lane 5), or 1.0 (lane 6) μg of T7RP autogene. Cell extracts were prepared, normalized for protein content, and analyzed for CAT activity.

Inducible T7RP activity in stable cell lines

HeLa cell lines expressing T7RP activity were generated by transfection and selection for cells bearing an episomal plasmid containing the T7RP gene under control of a dexamethasone (DEX)-inducible promoter (Mader and White, 1993; White *et al.,* 1994). T7RP activity (detected in 4 of 20 isolated cell lines) was tested under induced conditions by expression of CAT from a transfected P$_{T7}$/EMC/CAT *X*βgA$_{30}$ reporter gene after MVA infection (Fig. 4). Although MVA-infected HeLa cells expressed CAT activity only in the presence of an exogenous source of T7RP in the form of a cotransfected autogene (Fig. 4A, lanes 7 and 8), MVA-infected HeLa/GRE-T7RP cells exhibited increased CAT activity in the absence of an exogenous source of T7RP and specifically under induced conditions (Fig. 4B, lane 4). CAT activity under induced conditions (Fig. 4B, lane 4) was slightly higher than that derived from the autogene in the absence of induction (Fig. 4B, lane 7). However, cotransfection of the T7RP autogene did not further increase CAT activity in induced cells (Fig. 4B, lanes 4 and 8). T7RP activity as measured by CAT expression was regulated by varying the levels of induction as CAT activity correspondingly increased over the range of 0 to 250 nM DEX (Fig. 4C, lanes 1–6).

Comparison of T7RP-mediated activities

The three modes of introducing productive amounts of T7RP into cells (by MVA/T7RP infection, autogene transfection, or induction in HeLa/GRE-T7RP cells) were compared directly. HeLa cells or DEX-induced HeLa/GRE-T7RP cells were infected with either MVA or MVA/T7RP in the presence or the absence of the T7RP autogene (Fig. 5). Addition of the T7RP autogene inhibited expression in all cases except in HeLa cells infected with MVA, in which the autogene was the sole source of polymerase (Table 1 and Fig. 5). The highest activity was achieved with MVA/T7RP infection of HeLa cells not expressing an endogenous source of T7RP (Table 1 and Fig. 5, lanes 13 and 14). Expression in HeLa cells increased with increasing amounts of target DNA (Fig. 5, lanes 9–16, odd vs even lanes). Interestingly, HeLa/GRE-T7RP cells did not show such a dependence on P$_{T7}$ target DNA concentration (Fig. 5, lanes 1–8, odd vs even lanes).

Effect of α-amanitin

The T7 promoter has been shown to bind to eukaryotic nuclear factors and to act as a transcription start site for RNA polymerase II in eukaryotic cells (Sandig *et al.,* 1993; Bähring *et al.,* 1994). The fungal peptide toxin α-amanitin, which inhibits eukaryotic but not prokaryotic or vaccinia polymerases (Nevins and Joklik, 1977; Baroudy and Moss, 1980), was employed to investigate whether eukaryotic factors were involved in vaccinia virus MVA/T7RP expression. P$_{T7}$/EMC/CAT/*X*βgA$_{30}$ expression units were placed in the streamlined plasmid pMINI-S so as to reduce the potential for nonspecific transcription. The pMINI-S plasmids contain a limited amount (1812 bp) of DNA encoding an origin and an

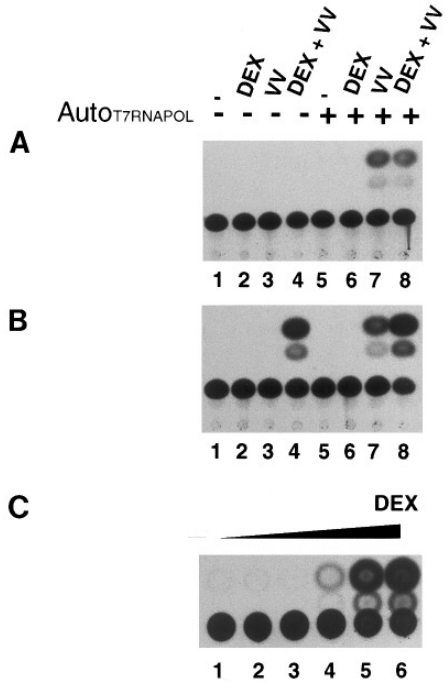

FIG. 4. Induction of T7RP activity in stable cell lines. (A) HeLa cells were transfected with 1 μg P$_{T7}$/EMC/CAT/*X*βgA$_{30}$ reporter DNA without (lanes 1–4) or with (lanes 5–8) 1 μg of T7RP autogene. Cells were either uninfected (lanes 1, 2, 5, and 6) or infected with MVA (lanes 3, 4, 7, and 8) under uninduced or induced conditions as indicated. Induction occurred with 25 nM DEX after the 6-h transfection period for an additional 24 h. CAT activity was measured and the resultant autoradiograph is shown. (B) HeLa/GRE-T7RP cells were treated identically as in A. (C) HeLa/GRE-T7RP cells induced with 0 (lane 1), 0.025 (lane 2), 0.25 (lane 3), 2.5 (lane 4), 25 (lane 5), or 250 nM (lane 6) DEX were transfected with 1 μg P$_{T7}$/EMC/CAT/*X*βgA$_{30}$ DNA. CAT assays were performed on an equivalent 1 μg of protein, for 45 min, the radiolabeled products analyzed by TLC, and the resultant autoradiograph is shown.

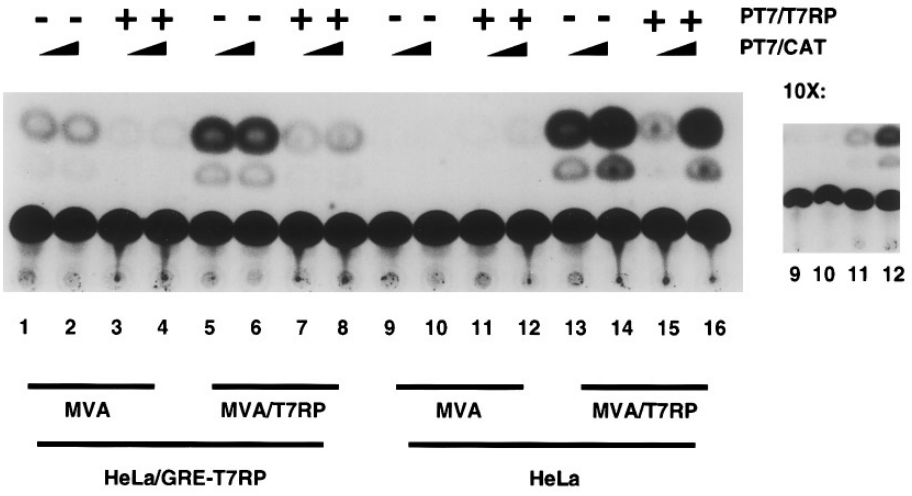

FIG. 5. Comparison of MVA/T7RP expression. Dexamethasone-induced HeLa/GRE-T7RP (lanes 1–8) or HeLa cells (lanes 9–16) were infected with 2 m.o.i. MVA (lanes 1–4, 9–12) or MVA/T7RP (lanes 5–8, 13–16) and cotransfected with or without 1 μg T7RP autogene as indicated with either 1 or 10 μg of P_{T7}/EMC/CAT/*X*βgA_{30} reporter (odd- and even-numbered lanes, respectively). Twenty-four hours after transfection, cells were lysed and analyzed for CAT activity using 100 ng lysate for 30 min. The insert shows samples 9–12 analyzed for CAT activity using 10 times more lysate (1 μg) for 30 min in order to visualize the activity. The resultant autoradiogram of separated products is shown.

ampicillin resistance gene as a backbone (Humphry *et al.,* 1996). To test the efficacy of introducing α-amanitin into eukaryotic cells via lipofection, the CAT gene under control of the eukaryotic CMV promoter was transfected into HeLa cells in the absence or the presence of α-amanitin peptide (Fig. 6A). Decreased CAT activity was observed in the presence of α-amanitin, consistent with its inhibitory activity on eukaryotic polymerases (Fig. 6A, lanes 1 and 2). In contrast, expression from the transfected pMINI-S/CAT construct was enhanced in the presence of α-amanitin (Fig. 6B, lanes 2–5) compared to its absence (Fig. 6B, lane 1).

EMC/CAT/*X*βgA_{30} expression units were also constructed under control of a wild-type T7 promoter, no T7 promoter, or a mutated T7 promoter. The modified T7 promoter was generated from a screen to isolate functional T7 promoters which have decreased ability to bind HeLa cell proteins in gel-shift assays (Lieber *et al.,* 1993b). Both the T7 wild-type and mutated promoters but not the promoterless CAT constructs produced CAT protein in coupled *in vitro* transcription/translation reactions in reticulocyte lysates (Fig. 7A, lanes 1–3). CAT constructs with a T7 wild-type promoter, lacking a T7 promoter or containing a mutated T7 promoter, were also transfected into HeLa cells in the absence or the presence of α-amanitin along with T7RNA polymerase in the form of a P_{T7}/EMC/T7RNP/*X*βgA_{30} autogene (Fig. 7B). The cells were then infected with MVA. The wild-type T7 promoter gave increased expression of CAT activity in the presence of α-amanitin compared to its absence, the difference being a sixfold increase when quantitated by phosphorimager analysis (Fig. 7B, lanes 1 and 4). Expression from the P_{T7}-less CAT construct was also enhanced in the presence of α-amanitin (Fig. 7B, lanes 2 and 5); however, the mutated T7 promoter showed only a threefold activation of CAT activity in the presence of α-amanitin (Fig. 7B, lanes 3 and 6).

DISCUSSION

The multicomponent vaccinia virus/T7RP eukaryotic expression system consists of a target gene (or genes) under control of a T7 promoter, the bacteriophage T7RP, and vaccinia virus-associated factors. The high transcriptional activity of T7RP coupled with vaccinia virus-enhanced expression of T7 transcripts allows high protein expression levels in cultured eukaryotic cells (Moss *et al.,* 1990; Ward *et al.,* 1995). We describe use of the vaccinia virus/T7RP system with a previously described replication-deficient vaccinia virus strain (Sutter and

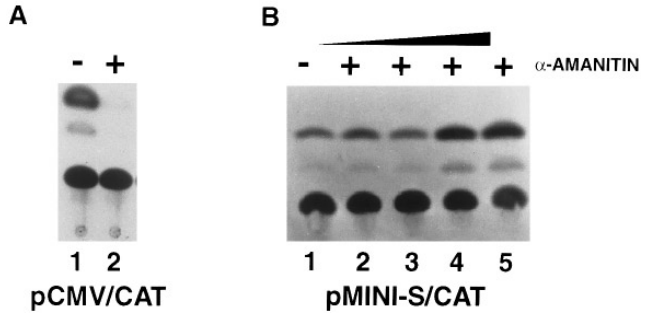

FIG. 6. Effect of α-amanitin on MVA/T7RP-mediated expression. (A) HeLa cells infected with 2 m.o.i. MVA were cotransfected with 1 μg pCMV[$-P_{T7}$]/CAT without (lane 1) or with (lane 2) 10 μg α-amanitin. Cell lysates were prepared and analyzed for CAT activity using 10 μg of protein for 2 h. The resultant autoradiograph is shown. (B) A pMINI-S plasmid containing P_{T7}/EMC/CAT/*X*βgA_{30} was transfected in MVA/T7RP-infected cells without (lane 1) or with 2 (lane 2) 5 (lane 3), 10 (lane 4), or 20 (lane 5) μg α-amanitin. Cell lysates were prepared and analyzed for CAT activity, with the resulting autoradiograph shown.

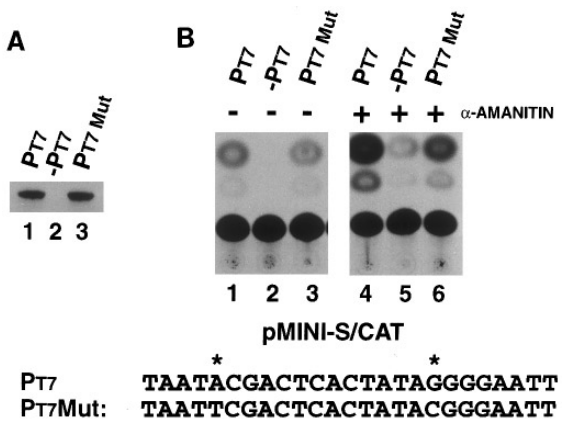

FIG. 7. Effect of α-amanitin on CAT expression from a wild-type T7 promoter, no T7 promoter, or a mutated T7 promoter. (A) pMINI-S plasmids containing EMC/CAT/*X*βgA$_{30}$ under control of a T7 promoter (lane 1), no T7 promoter (lane 2), or a mutated T7 promoter (lane 3) were used to prime *in vitro* transcription/translation reactions. The radiolabeled proteins were separated by SDS–PAGE and visualized by autoradiography. (B) HeLa cells were infected with 2 m.o.i. of MVA and cotransfected with 1 μg of T7RP autogene and 1 μg each of pMINI-S plasmid containing EMC/CAT/*X*βgA$_{30}$ under control of a T7 promoter (lanes 1 and 4), no T7 promoter (lanes 2 and 5), or a mutated T7 promoter (lanes 3 and 6) in the absence or the presence of 10 μg α-amanitin as indicated. Cell extracts were analyzed for CAT activity using 1 μg protein for 30 min as described and the resultant autoradiograph is shown. The CAT assays shown in B were quantified by phosphorimaging (see text). The sequences of both wild-type and mutated T7 promoter regions are shown with stars indicating the sites of mutation.

Moss, 1992). The MVA strain has yielded high expression with reduced cytopathic effects and allowed analysis of expression independent of viral replication (Sutter *et al.,* 1995; Wyatt *et al.,* 1995). We find, as previously observed (Sutter *et al.,* 1995; Wyatt *et al.,* 1995), that MVA/T7RP-mediated expression allows high expression, despite being replication deficient. In addition, these results were achieved with the relatively low multiplicity of 2 infectious units. At this level, high expression was observed for the CAT gene with both CMV/P$_{T7}$ and P$_{T7}$ expression units, with the dual CMV/P$_{T7}$ promoter giving the highest overall expression (440-fold over the CMV promoter alone).

T7 RNA polymerase from nonviral templates

Two methods were employed to control the amount of T7RP activity in the vaccinia virus/T7RP expression system apart from varying MVA/T7RP infection: (i) varying the amounts of a self-amplifying T7RP autogene or (ii) modulating the levels of T7RP induction in dexamethasone-regulatable stable cell lines. Transfection of low levels of autogene DNA and expression from induced HeLa/GRE-T7RP cells both yielded far higher CAT expression than that achieved by the CMV promoter. Use of pCMV[−P$_{T7}$]/T7RP as a "primer" in the autogene system did not improve CAT expression over that obtained with the autogene alone. The option of dispensing with such a primer plasmid is fortunate, as pCMV[−P$_{T7}$]/T7RP expression requires nuclear localization and thus is inherently limiting in the cytoplasmic vaccinia virus expression system.

Increasing T7RP activity by either autogene addition or induction in HeLa/GRE-T7RP cells yielded quantitive increases in reporter gene expression, independent of increases in viral factors. However, elevating autogene levels beyond a certain point eventually inhibited expression, and autogene addition to cells already expressing T7RP from MVA/T7RP infection, or from an inducible GRE-T7RP plasmid, either did not further increase or significantly inhibited expression. Indeed, an apparent limit appears to exist above which increasing T7RP levels independent of the virus does not yield a concomitant increase in target gene expression. A potential explanation for this is that, as in prokaryotes (Tabor and Richardson, 1985; Studier and Moffatt, 1986), high levels of T7RP and its promoter are toxic. Therefore, it may be advantageous to deliver amounts of T7RP just sufficient so as not to be limiting. Overall, viral delivery of T7RP yielded the highest amounts of CAT gene expression at the infection levels utilized.

Transcriptional vs translational mechanisms

Stimulation of T7RP-directed expression in eukaryotic cells by vaccinia virus appears to be transcriptional since: (1) expression is stimulated by vaccinia virus in the presence of an inhibitor of DNA replication (AraC), eliminating plasmid DNA amplification as a factor (Elroy-Stein and Moss, 1990); (2) Northern analysis shows T7RP levels increase during vaccinia infection (Elroy-Stein and Moss, personal communication); and (3) CAT expression from transfected EMC/CAT/*X*βgA$_{30}$ RNA is not stimulated by vaccinia virus infection, arguing against a posttranscriptional mechanism (this report). In our study, it remains possible that expression from exogenously added EMC/CAT/*X*βgA$_{30}$ RNA was not efficiently coupled to translation pathways *in vivo,* similar to effects shown with other expression systems using heterologous components (Lewicki *et al.,* 1993; Iost and Dreyfus, 1994, 1995; Chao *et al.,* 1995). As concerns the effects of vaccinia virus on T7 transcription, these may be direct, such as through the activity of viral polymerase and associated factors, or indirect, such as through optimal localization of the transfected plasmid with viral or cellular components.

Hybrid characteristics of the expression system

By measuring CAT reporter activity with or without T7 promoter control, in combination with virus either encoding or not encoding T7RP, it could be shown that MVA/T7RP-mediated expression is truly a hybrid system, consisting predominantly of a T7RP-mediated component, but also of a MVA-associated component. The T7RP

activity is P_{T7} dependent, while the MVA-associated component is P_{T7} and T7RP independent. MVA-associated activity may reflect transcription from otherwise cryptic start sites by vaccinia virus-derived polymerase and associated factors, possibly as has been reported for the CAT coding sequence (Ryan, 1992), as well as high translation efficiencies for RNAs with a 5′ EMC IRES. Unlike translation initiation of capped eukaryotic transcripts, the EMC IRES enhances translation of juxtaposed downstream genes regardless of its position within RNA and independent of 5′ free ends (Chen and Sarnow, 1995). In addition, MVA-encoded factors are likely to activate the T7RP autogene, which initiates expression in the presence of MVA infection but absent other sources of T7RP. Theoretically, since only one polymerase molecule is able to initiate the amplification process, the autogene is a sensitive measure of transcription in infected eukaryotic cells.

Interference by eukaryotic factors

The opposite effects of α-amanitin in inhibiting pCMV[$-P_{T7}$]/CAT activity but increasing MVA/T7RP-mediated CAT expression suggest that the α-amanitin peptide acts by discriminating between eukaryotic and non-eukaryotic transcription in the hybrid system. Since T7RP-mediated CAT expression was not inhibited by α-amanitin, expression appears due to phage polymerase and vaccinia factors as opposed to α-amanitin-sensitive eukaryotic factors. The increase in expression from P_{T7}/CAT and P_{T7}-less/CAT plasmids by α-amanitin suggests that both T7RP-mediated and MVA-associated expression may be susceptible to eukaryotic factors repressing expression; however, α-amanitin-induced alterations in viral action cannot be excluded. The former interpretation is supported by reports showing (1) HeLa cell nuclear factors bind T7 plasmids (Sandig *et al.,* 1993); (2) the T7 promoter acts as a RNA polymerase II start site (Bähring *et al.,* 1994); and (3) eukaryotic RNA polymerase II interferes with expression from downstream promoters through which it transcribes (Cullen *et al.,* 1984; Proudfoot, 1986; Corbin and Maniatis, 1989). The increase in P_{T7}/CAT activity caused by α-amanitin might then be due to the clearing of eukaryotic polymerase and/or associated factors from the T7 plasmid. This would allow increased expression from T7RP and MVA-associated factors. Consistent with this, we report that a mutated T7 promoter which is less able to bind HeLa nuclear factors (Lieber *et al.,* 1993b) appears less stimulated by α-amanitin. Understanding the effects of repressive eukaryotic factors may lead to methods of further enhancing vaccinia virus/T7RP-mediated expression in eukaryotic cells.

MATERIALS AND METHODS

Plasmid construction

Using VENT polymerase (New England Biolabs) and standard thermocycling procedures to add restriction enzyme sites suitable for cloning, the CAT gene was subcloned into the *Hin*dIII/*Xho*I sites of pCMV.3 (Orgryzko *et al.,* 1994), a eukaryotic expression vector containing both a CMV and a T7 promoter (P_{T7}), which is referred to as pCMV[P_{T7}]/CAT. The CAT gene was similarly subcloned into the *Hin*dIII/*Xho*I sites of pCMV[$-P_{T7}$], which is identical to pCMV[P_{T7}] except for deletion of P_{T7}. The CAT gene was also subcloned into the *Nco*I/*Xho*I sites of the pCITE-2a vector (Novagen) modified to contain the *Xenopus laevis* β-globin 3′ untranslated region (UTR) from pEMCLuc*X*βgA$_n$ (Deng *et al.,* 1991), a gift from J. Wolff, University of Wisconsin) followed by a polynucleotide tract of 30 adenine residues (*X*βgA$_{30}$). The pCITE-2a vector as supplied contains a T7 promoter and an IRES for eukaryotic ribosomes derived from the EMC virus 5′ UTR. The CAT gene flanked by the EMC 5′ UTR from pTM1 (Moss *et al.,* 1990; a gift from B. Moss, National Institutes of Health) and the *X*βgA$_{30}$ 3′ UTR was also placed under a T7 promoter, no T7 promoter, or a modified T7 promoter (Lieber *et al.,* 1993a,b) and this expression unit was subcloned into the pMINI-S plasmid (Humphry *et al.,* 1996) modified to contain the multiple cloning site from pUC19. The pMINI-S T7 expression units contain the *lac* operator originally derived from pET-21(+) followed by a stem-loop structure previously shown to enhance RNA stability (Fuerst and Moss, 1989).

The T7RP autogene was made using thermocycling procedures to add restriction enzyme sites to the T7RP gene derived from pAR1173 (American Tissue Type Culture 39562). An extra codon (GGA) was added following the initiating ATG so as to provide an *Nco*I site. Therefore, the expressed protein contains an added glycine residue following the initial methionine. Modification of the T7RP amino terminus appears not to effect its activity adversely (Dunn *et al.,* 1988). The T7RP insert was subcloned into the *Nco*I/*Xho*I sites of the pET-21(+) vector (Novagen) modified to contain the EMC 5′ UTR, including a 5′ stem-loop structure (Fuerst and Moss, 1989), and the *X*βgA$_{30}$ 3′ UTR. This plasmid retains the T7*lac* promoter/operator and *lacI* gene required for repression during propagation in bacteria and a T7 terminator. The T7RP gene was also subcloned into the *Hin*dIII/*Xho*I sites of pCMV[$-P_{T7}$] and the *Nco*I/*Xho*I sites of pCITE-2a (Novagen). The functionality of both CAT and T7RP T7 expression units was tested using coupled *in vitro* transcription/translation reactions in reticulocyte lysates (Promega) with [^{35}S]methionine (New England Nuclear). Reactions were subjected to SDS–PAGE and visualized by fluorography to detect the expected protein products.

Propagation of plasmids containing the T7RP gene under control of its own promoter (T7RP autogene) is toxic to bacteria and strongly selects for nonfunctional forms (Dubendorf and Studier, 1991). Bacteria bearing functional T7RP autogenes were viable only under conditions when T7RP RNA expression was repressed by the *lacI* gene product and T7RP protein activity was inhibited by T7 lysozyme (Dubendorf and Studier, 1991). *lacI* repression alone was sufficient for the generation of

functional P_{T7}/EMC/T7RP autogenes (i.e., the presence of T7 lysozyme as expressed from pLysS or pLysE plasmids (Dubendorf and Studier, 1991) was not necessary (data not shown). This relaxed stringency may be due to the presence of the EMC 5′ IRES which may inhibit production of functional polymerase in prokaryotes and lower the requirement for repression of autogene activity.

Vaccinia virus infection and liposome-mediated transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) containing 10% (v/v) fetal bovine serum (FBS) (Hyclone) in humidified air, 5% CO_2 at 37°C. For transfections, cells were seeded in 24-well plates at 300,000 cells per well. Cells were washed with reduced serum media (OPTI-MEM; Life Technologies, Inc.) and unmodified MVA virus or recombinant MVA expressing T7RP (gifts of L. Wyatt and B. Moss, National Institutes of Health) was added at indicated infection levels in 200 μl OPTI-MEM/well for 30 min. During the infection period, DNA and lipid transfection reagents were mixed and incubated for 30 min at room temperature. DNA was purified by CsCl double-band ultracentrifugation (Fordis and Howard, 1987). Ratios of DNA:lipid of 1:2.5 or 1:5 (w/w) with LipofectACE or DMRIE-C (Life Technologies, Inc.) were used in 1 ml OPTI-MEM. After the infection period, the cells were washed and the DNA/lipid precipitates were added for 6 h. The lipofection mixes were washed away and cells were harvested 24 h postinfection initiation. Gene expression was measured in the cell lysates by assaying for CAT activity (Fordis and Howard, 1987). CAT activity was quantitated by thin-layer chromatography and phosphorimaging. If conversion of greater than 80% of chloramphenicol occurred, the assay was considered out of the linear range and lysates were diluted appropriately for quantification. The extreme sensitivity of the CAT assay allowed readily detectable expression with an infectivity of only 2 multiplicities of infection (m.o.i.), an amount that would not be expected to infect all cells but allowed a reduction of viral side effects.

RNA preparation and transfection

Plasmids containing the CAT gene flanked by a 5′ EMC and a 3′ *X*βgA_{30} UTR and preceded by a T7 promoter were linearized with *Bgl*II which cleaves at the terminal 3′ adenine residue in the artificial A_{30} tract. Uncapped RNA was transcribed *in vitro* using T7RP (MEGAscript High Yield Transcription Kit; Ambion, Inc.) and isolated by LiCl precipitation. For RNA transfections, HeLa cells were infected and transfected as described above except the ratio of RNA:LipofectACE was 1:2 (w/w) using 1 μg CAT RNA with 3 μg tRNA as carrier in 1 ml OPTI-MEM. The cells were harvested 24 h postinfection initiation.

Generation of stable cell lines

The pGRE5-1/EBV plasmid (Mader and White, 1993; White *et al.,* 1994; a generous gift from J. H. White, McGill University, Montreal) is an Epstein–Barr virus (EBV)-based episomal mammalian expression vector containing the EBV nuclear antigen (EBNA) gene and a series of five glucocorticoid response elements (GRE) which activates transcription in the presence of dexamethasone (DEX). A *Bam*HI fragment containing the T7RP gene was excised from pAR1173 (American Tissue Type Collection 39562) and oriented appropriately into the *Bam*HI site of pGRE5-1/EBV. DNA was purified by anion-exchange chromatography (QIAGEN, Inc.) and HeLa cells were transfected by electroporation as described (Goldstein *et al.,* 1989) except without the addition of sodium butyrate. Cells were incubated in DMEM/10% FBS for 48 h after transfection before selection was initiated in 250 μg/ml hygromycin (Boehringer Mannheim Biochemicals) with the media changed every 2–3 days. After 4 weeks of selection, cell colonies were isolated and expanded.

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